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MICRO-MILLING ENHANCES IRON BIOACCESSIBILITY FROM WHOLEGRAIN WHEAT

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Abstract

Cereals constitute important sources of iron in human diet; however, much of the iron in wheat is lost during processing for the production of white flour. This study employed novel food processing techniques to increase the bioaccessibility of naturally-occurring iron in wheat. Iron was localized in wheat by Perl's Prussian blue staining. Soluble iron from digested wheat flour was measured by a ferrozine spectrophotometric assay. Iron bioaccessibility was determined using an *in vitro* simulated peptic-pancreatic digestion, followed by measurement of ferritin (a surrogate marker for iron absorption) in Caco-2 cells. Light microscopy revealed that iron in wheat was encapsulated in cells of the aleurone layer and remained intact after *in vivo* digestion and passage through the gastrointestinal tract. The solubility of iron in wholegrain wheat and in purified wheat aleurone increased significantly after enzymatic digestion with driselase, and following mechanical disruption using micro-milling. Furthermore, following *in vitro* simulated peptic-pancreatic digestion, iron bioaccessibility, measured as ferritin formation in Caco-2 cells, from micro-milled aleurone flour was significantly higher (52%) than from whole aleurone flour. Taken together our data show that disruption of aleurone cell walls could increase iron bioaccessibility. Micro-milled aleurone could provide an

alternative strategy for iron fortification of cereal products.

Key words: bioaccessibility, micro-milling, wheat, aleurone

Introduction

Iron deficiency (ID) and iron deficiency anemia (IDA) are nutritional disorders affecting large population groups world-wide[1]. These disorders are prevalent in developing countries and fortification of foods with iron has proved to be an effective strategy to combat deficiency. However, food fortification remains a major challenge since water soluble fortificants change the colour and taste of foods and less soluble fortificants, such as ferric pyrophosphate or elemental iron powder, cause fewer sensory changes in foods but are poorly absorbed in the gastrointestinal (GI) tract[2,3]. Consequently, the development of novel approaches, which both improve iron bioavailability and are acceptable to consumers, may provide an effective solution to the current problems of iron fortification.

Cereal grains and cereal products constitute important sources of iron in human diet in many countries (40-50% total daily intake in the UK, (NDNS 2014)[4]. Iron in wheat is confined in the aleurone layer (AL), a single layer of cells located between the endosperm and outer pericarp of the wheat grain[5]. This layer is removed as part of the bran component during the production of white flour, hence the mandatory fortification of white and brown flours with elemental iron powder iron (1.65 mg / 100 g flour) in the UK. However, this iron source has low bioavailability[6]. Furthermore, the fortification of

flour with iron has additional challenges due to the presence of high levels of dietary inhibitors such as phytates, tannins and dietary fibre (e.g. anionic polysaccharides such as pectins), which have the potential to interact with iron and reduced bioavailability[7].

The aim of the current study is to determine whether the bioaccessibility of endogenous iron in wheat can be increased by micro-milling of wheat products and in particular the AL since it contains approximately 70% of the iron in wheat grain[8]. In essence, this process employs mechanical disruption of wheat to rupture the cell walls comprising the AL and thus expose the intra-cellular contents. Remarkably, particle size reduction enhanced iron bioavailability from both elemental iron and iron nanocompounds[9, 10]. We hypothesize that this process will increase bioaccessibility of iron from aleurone and thereby enhance iron bioavailability. We propose that micro-milled aleurone could provide a bioavailable source of iron for use in food fortification.

Materials and Methods

Reagents and chemicals

Unless otherwise stated, all the reagents and chemicals used in this study were purchased from Sigma-Aldrich Company Ltd (Dorset, UK). Driselase (EC286-055-3), pepsin (EC232-629-3) and pancreatin (EC232-468-9) were stored at-20 °C. Solutions of enzymes were all prepared freshly just before use.

Wheat samples

Purified aleurone flour and micro-milled aleurone and wholegrain wheat flour were a gift from Bühler AG (Switzerland). Standard ball-milled aleurone product has an average particle size of 110-240 µm, while that of the micro-milled is 10-20 µm, which is ~3 times smaller than the average diameter of aleurone cells (60 µm)[11,12]. Micro-milling was performed using a roller mill (Micromill; Bühler AG, Switzerland). Wholegrain wheat flour was obtained from *Triticum* durum L. wheat grain ground in a blender (Millbo Italy, Svevoc.v.).

Moisture analysis

Moisture content of the samples was determined according to the AOAC (1999) method. Briefly, samples were weighed and placed in an oven at 100 °C overnight to dry for 24-48 h until constant weights were achieved. Afterwards the percentage moisture content was calculated for each sample.

Determination of iron content in wheat samples

Wheat samples were weighed in crucibles with lids. The samples were dried in an oven at 70°C overnight and cooled in a desiccator. Samples were charred over a Bunsen burner flame at a low heat to eliminate smoke before placing in a muffle furnace at 525°C for 3 h hours during which all the organic matter was oxidized leaving remnants of clean white ash. Samples were oven-dried for 48 hours, cooled in a desiccator and reweighed. Fe, Mg, Zn, Ca and Mn concentrations in the samples was analysed using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Thermo-Fisher).

Plasma parameters and sample aspiration methods were performed according to the manufacturer's recommendations. Mineral concentrations were extrapolated from the standard curve in the range of 0.1 –10 µg/ml. The internal standard, Yttrium (Merck Millipore), was added to each sample according to manufacturer's specification to correct for sample losses due to volatility and evaporation.

***In vitro* peptic-pancreatic digestion**

Samples were digested by simulated peptic-pancreatic digestion[13]. Enzymes and bile extract were demineralized with Chelex-100 (Bio-Rad Laboratories Ltd., Hercules, CA) before performing the experiments. The weight of samples used for experiment was calculated according to iron content in different samples to ensure that equal amounts of iron (150 µg) were used for digestion experiments. Following this, known weights of samples (in quadruplicate) were added to 10 mL of isotonic saline solution (140 mM NaCl and 5 mM KCl) and were adjusted to pH 2.0 with HCl (1 M). During peptic digestion, 0.5 mL pepsin (16 mg/mL) was added and incubated at 37 °C for 75 min followed by pH was adjustment to 5.5 with $NaHCO_3$ (1 M) to stop peptic digestion. Afterwards, 2.5 mL bile-pancreatin extract (8.5 mg/mL bile extract and 1.4 mg/mL pancreatin) was added and pH was adjusted to 7.0 with $NaHCO_3$ (1 M) to start pancreatin-bile digestion. The volume was brought to 15 mL by adding isotonic saline solution and incubated at 37 °C for 120 min. Following digestion, tubes were centrifuged at 3000 x g for 5 min and the supernatant of digests was retained for experiment.

Cell culture

Caco-2 cells (ATCC; HTB-37) at passage 28 were used for the experiments. Cells were grown in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Life Technologies, UK), which contained 1% antibiotic/antimycotic solution, 25 mM HEPES and 10% fetal bovine serum. For the experiment, cells were trypsinised and seeded into 6-well plates at a density of 50,000 cells in 2.5 mL DMEM. Cells were incubated at 37 °C with 5% CO_2 and 95% air for 14 days while the medium was changed every 2 days.

The day before experiments, DMEM was replaced with minimum essential medium (MEM, Gibco Life Technologies, UK) containing 10 mmol/L PIPES, 1% penicillin and streptomycin, 11 µM dexamethasone and 0.87 µM insulin and the cells were incubated at 37 °C for 24 h. Afterwards fresh MEM (2 ml) was added to the cells. 1.5 mL of each digest was pipetted into cellulose dialysis tubing (15,000 Da molecular weight cutoff dialysis membranes (Tubing Spectra/Por 7 dialysis membrane, Fisher Scientific) that were exposed to the medium bathing the cells. Cells were then incubated at 37 °C for 2 h for iron uptake. The baseline control was incubated with only MEM medium. Following that, the digest was removed, 1 mL of supplemented MEM was added to the cells and these were incubated for a further 22 h. Following this incubation period, cells were washed with PBS and lysed with Mammalian Protein Extraction Reagent (MPER®, Thermo Fisher Scientific, Cramlington, UK). The cell lysate was centrifuged (5 min, 16,000 x g) to remove cell debris and the supernatant used for ferritin and protein analysis. Thereafter, cells were harvested in 200 mL PER protein lysate solution (Thermo

Scientific) and analyzed for ferritin content using a commercially available ELISA (Ramco Laboratories, TX, USA). Experiments were carried out in triplicate and data expressed as ng ferritin per mg cell protein.

***In vitro* digestion with driselase**

Wholegrain wheat flour was digested with driselase (EC286-055-3) an enzyme mixture containing laminarinase, xylanase and cellulase activity that hydrolyses cell walls of plants. Two milliliters of enzyme solution (1 Unit/mL) was made by mixing 100 µL of 2% driselase (w/v) with 1.9 mL buffer (1.33 mL of 50 mM sodium acetate and 0.57 mL of 50 mM acetic acid, pH 2.5). 100 mg of wholegrain wheat flour was added to 2 mL enzyme solution and this was incubated at 37 °C for 6 h. The control group was incubated with the buffer without driselase. After digestion, tubes were centrifuged at 3000 x g for 5 min and the supernatant was saved for measurement of soluble iron afterwards.

Iron Solubility from Wheat samples

The amount of soluble iron was analyzed using the ferrozine assay[14]. The blank solution consisted of enzymes without the wheat samples. One milliliter of supernatant from each of the digests, the blank and the standards were added to microfuge tubes (Figure 2). To this was added 0.1 mL of solution containing 10% HCl (v/v) and 5% hydroxylamine hydrochloride (w/v), mixed and incubated at room temperature for 30 min. Afterwards, 0.1 mL of a solution containing 5 mg/mL ferrozine and 1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) buffer was added, mixed

and allowed to stand at room temperature for 1 h. Finally, the absorbance of the solutions was measured in a spectrophotometer (Camspec M330 UV-visible) at 562 nm. Standard solutions containing 0.015 – 0.5 µg/mL $FeCl_3$ were prepared and treated as with the samples.

Microscopic Localization of Iron in Wheat Aleurone Layer

The localization of iron and the structure of aleurone layer observed under the microscope after staining with Perl's Prussian blue according to a protocol described by Wang and Cuschieri[15]. The stain used was a mixture of 2.5% potassium ferrocyanide (w/v) in 2.5% hydrochloric acid (HCl) (v/v). Approximately 50 mg of wheat sample and 500 µL of Perl's Prussian blue solution were placed in 1.5 ml microfuge tubes and incubated at room temperature for 30 min before examination with a light microscope (Axioskop 2 mot plus, Car-Zeiss, UK) to reveal tissue structure and iron deposits in the aleurone layer.

Animal studies

Changes to the structure of aleurone layer and the iron content of the aleurone cells in different regions of the gastrointestinal tract and in faeces were also determined. Eight male six weeks old C57BL/6 mice (Charles Rivers, Kent, U.K.) were used for the studies. Mice were fasted overnight and then fed in two groups of four mice wholegrain wheat flour or aleurone flour ad libitum for 24 h to allow complete transit through the gastrointestinal tract. Mice were housed in a light- and temperature-controlled room

with ad libitum access to deionized water.

Following feeding flour for 24 h, mice were killed by cervical dislocation and contents of the stomach, duodenum, jejunum, ileum and colon contents as well as faeces from the mice were removed and placed in tubes for microscopic examination. Perl's Prussian blue staining and light microscopic examination in each sample was performed as described above. All procedures were conducted in accordance with methods approved by the United Kingdom Animals (Scientific Procedures) Act 1986.

Statistical Analysis

Data were analysed with Microsoft Office Excel 2010 and SPSS software 20.0.0 (SPSS Inc., USA). Data are shown as mean ± SEM. Comparison of means was analysed either by Student's unpaired t-test, or one-way analysis of variance (ANOVA) with Turkey's post-test for multiple comparisons. Significant differences were considered at $P<0.05$.

Results

Mineral analysis in wheat samples

Table 1 shows the mineral content of whole grain wheat and aleurone samples. Mineral concentrations were significantly enriched in the aleurone fraction compared with the whole wheat samples, with iron in particular being some 3-4 fold enriched. Mineral content of wholegrain and aleurone flour was not significantly altered following micro-milling.

Microscopic localization of iron in wheat products

Light microscopy revealed the localization of iron in the aleurone layer of whole wheat (Fig 1). Aleurone cells were largely resistant to *in vivo* digestion during transit along the gastrointestinal tract. Microscopic examination of digests from mice that were fed aleurone flour overnight revealed iron-stained globules encased within aleurone cells obtained from different regions of the gastrointestinal tract (Fig 2a-e) and also in fecal contents (Fig 2f).

***In vitro* iron solubility from aleurone samples**

The aleurone cell walls were disrupted by enzymatic digestion with driselase (Fig 3a and 3b) and by micro-milling (Fig 3c and 3d). Next we investigated whether enzymatic or mechanical disruption of aleurone cells would alter bioaccessibility of iron. Following driselase treatment (Fig 4a) and mechanical disruption through micro-milling there was a significant increase in iron solubility in aleurone and whole wheat samples (Fig 4b).

Micro-milling and iron availability in wheat aleurone

To determine whether mechanical disruption of the aleurone cell layer increased iron solubility and in turn lead to increased iron bioaccessibility, we employed a model of *in vitro* digestion/cell iron uptake[13]. Micro-milling of purified aleurone flour and wholegrain wheat flour (Fig 5) significantly enhanced iron bioaccessibility after peptic-pancreatin digestion of the samples and iron uptake in Caco-2 cells, using cell ferritin protein content as a surrogate marke**r.**

Discussion

Fortification of staple crops with iron is recommended for alleviation of the high prevalence of ID and IDA in population groups in many countries[16]. Well established strategies employ ferrous (II) salts, ferric (III) salts, ferric (III) chelates or elemental Fe powders as the primary fortificants[17]. Moreover iron supplementation poses inherently difficult issues such as solubility, bioavailability, toxicity or tolerability in the gastrointestinal (GI) tract[18,19]. Consequently, transgenic transformation of cereals and other food crops became an attractive option for improving iron nutrition in human populations[20,21]. Plants have been genetically modified to yield grains that express ferritin[22], phytase[23,24], haemoglobin[25,26] or co-expression of ferritin and phytase in an attempt to improve iron nutrition[27]. Bio-fortification, both is still in its developmental phase and is beset by numerous challenges both technical and emotive. Thus, if a simple food processing technique could increase iron bioaccessibility, or a naturally iron-rich food component could be modified to provide a bio-accessible and bioavailable source of iron for food fortification, this would represent a major advance in human nutrition.

Here we have investigated whether wheat aleurone might provide a bioaccesible source of iron. One immediate obstacle to the use of aleurone is that the cell walls, which are composed of mainly non-starch polysaccharides (dietary fiber), are highly resistant to digestion in the upper GI tract of humans and many experimental animals. Microscopic examination of the lumenal contents from different segments of mouse (GI) tract and even from fecal samples revealed encapsulated iron in intact aleurone cells (Fig 2). This

suggests that iron in aleurone cells is partly accessible even after transit throughout the entire length of the GI tract. It has been reported that plant cell walls are resistant to digestion in the upper GI tract of humans[28] with only 60% of the aleurone cell walls degraded in this region in the pig[29]. Colonic fermentation could lead to further degradation of cell walls and the release nutrients in the distal segment of the GI tract. A study using animal models demonstrated that 45% and 24% of aleurone was degraded during fermentation in the colon of rats and cockerels, respectively[30]. Moreover, partial degradation of the aleurone cell walls was evident in fecal samples from rats[31] fed wheat fractions. However, for iron at least, little or no absorption takes place in the colon[32,33].

Clearly to be of use as a food fortificant the aleurone cell walls would need to be disrupted to provide increased access to the iron contained within. Our first approach was to use an enzymatic digest with driselase. Following a 6 h digest with driselase there was a significant increase in the release of soluble iron (Fig 4a). While this approach increases iron release from aleurone cells, its use would be limited to situations where pre-digested aleurone could be added as a food fortificant. We therefore also used a non-enzymatic approach by mechanically distrupting aleurone through micro-milling. Micro-milling of the AL was found to significantly increase iron solubility and bioaccessibility (Figs 4b and 5). The increase in bioaccessibility might be due to increased digestibility and degradability by grinding, which increases the surface area of the samples for enhanced enzymatic digestion[34]. Furthermore, there is evidence that phytic acid, a resident component of the AL, is decreased during ball milling[35]. Phytic

acid is a potent inhibitor of non-haem iron bioavailability[13,36]. It is possible that changes in phytate species and concentration as a result of micro-milling may influence iron bioaccessibility from aleurone flour and this is currently under investigation. Moreover, particle size reduction increased redistribution of cellulose rich fibre fractions (another potent resident inhibitor of iron absorption) in favour of water soluble fibre components as well as increased gastrointestinal function[37].

An advantage of the mechanical approach to iron release from wheat aleurone is that the bioaccesibility of the endogenous aleurone iron reservoir could be increased through modified food processing technique. Potentially this could enhance the bioaccessibility of iron from wholegrain flour. Furthermore, micro-milled aleurone could offer a natural, stable, bioavailable iron fortificant or complement in foods. While our study has focused on iron, micro-milled aleurone could potentially provide a bioavailable source of a number of other minerals (e.g. calcium) and vitamins (e.g. thiamine, nicotinic acid and folate), all of which are commonly added as fortificants to white wheat flour[38-40]. Indeed aleurone has been studied previously as a potential folate source for incorporation into bread. This approach would also be feasible for iron fortification. Published data already shows that wheat bread made with white flour enriched with 20% aleurone has a flavour similar to standard white bread and contains comparable levels of nutrients to wholegrain bread[41]. Subsequent studies will address the optimal particle size and relative proportions of micro-milled aleurone enrichment required to achieve comparable or improved iron absorption efficacy to standard

inorganic iron fortificants. This strategy might contribute to an improvement of the management of iron status in vulnerable groups in different countries. While our *in vitro* data support the notion that micro-milled aleurone might be useful as an iron fortificant, it will be important to validate our findings *in vivo* by assessing the bioavailability of iron from aleurone-enriched wheat products in human volunteers, both in single meal studies and also as part of more complex diets.

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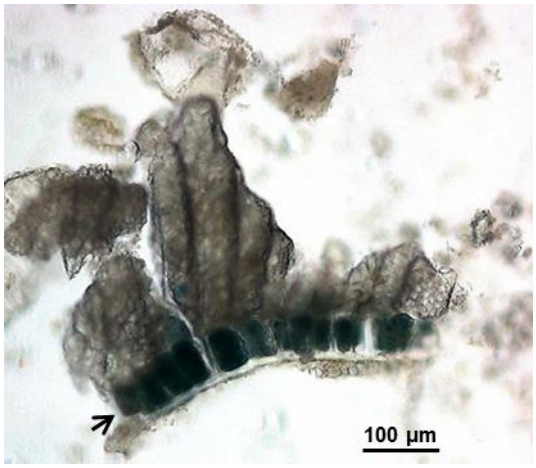

Fig 1. Localization of iron in the aleurone layer of whole wheat flour. Wheat flour treated with Perl's Prussian Blue solution and visualized under the microscope revealed a single blue stained aleurone layer (arrow).

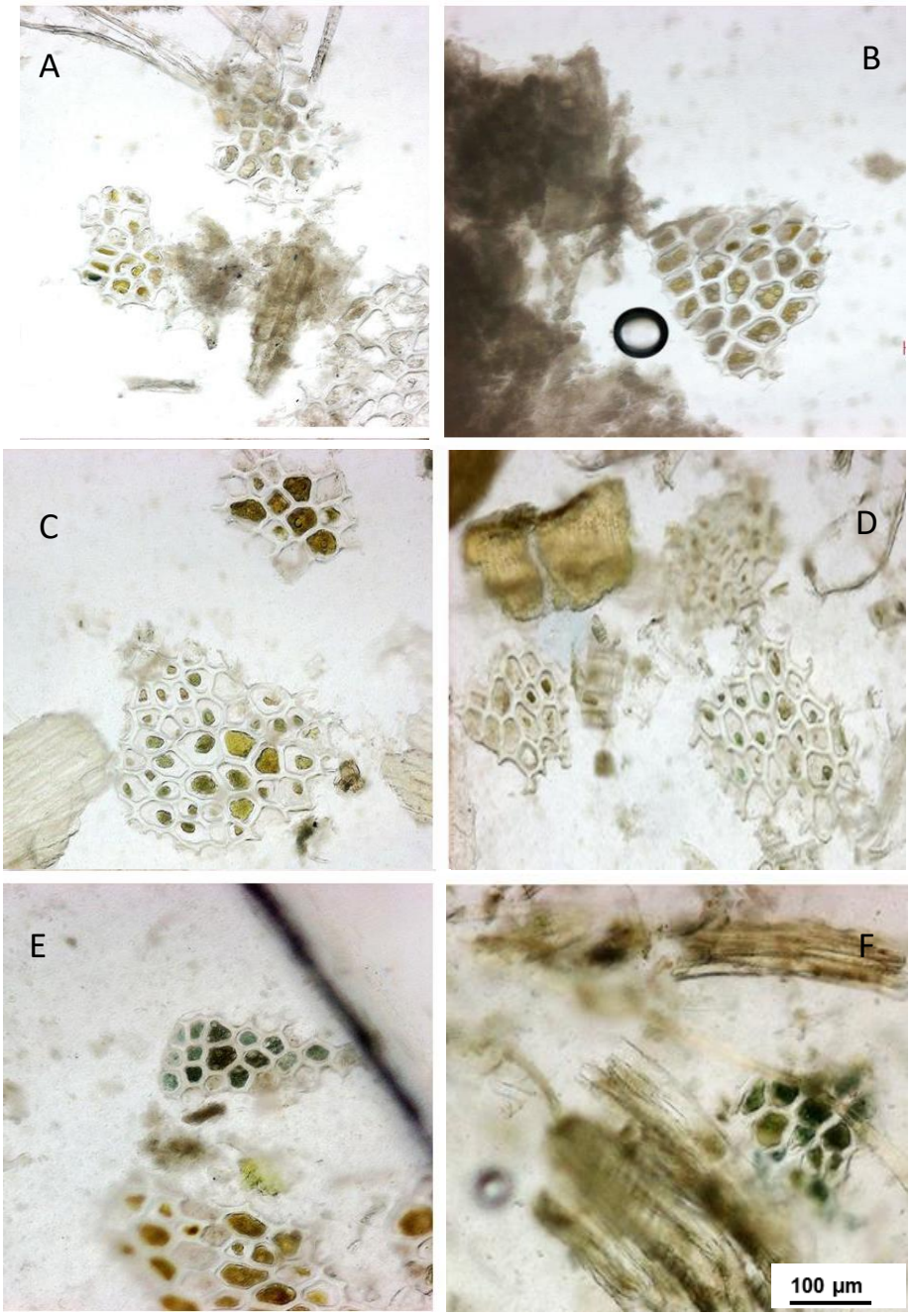

Fig 2. Structure and localization of iron in aleurone flour after passing through gastrointestinal tract of mice. Mice were fed aleurone flour overnight and food contents were obtained from different parts of the gastrointestinal (GI) tract for microscopic observation. Samples were obtained from stomach (A), duodenum (B), jejunum (C), ileum (D), colon (E) and feces (F).

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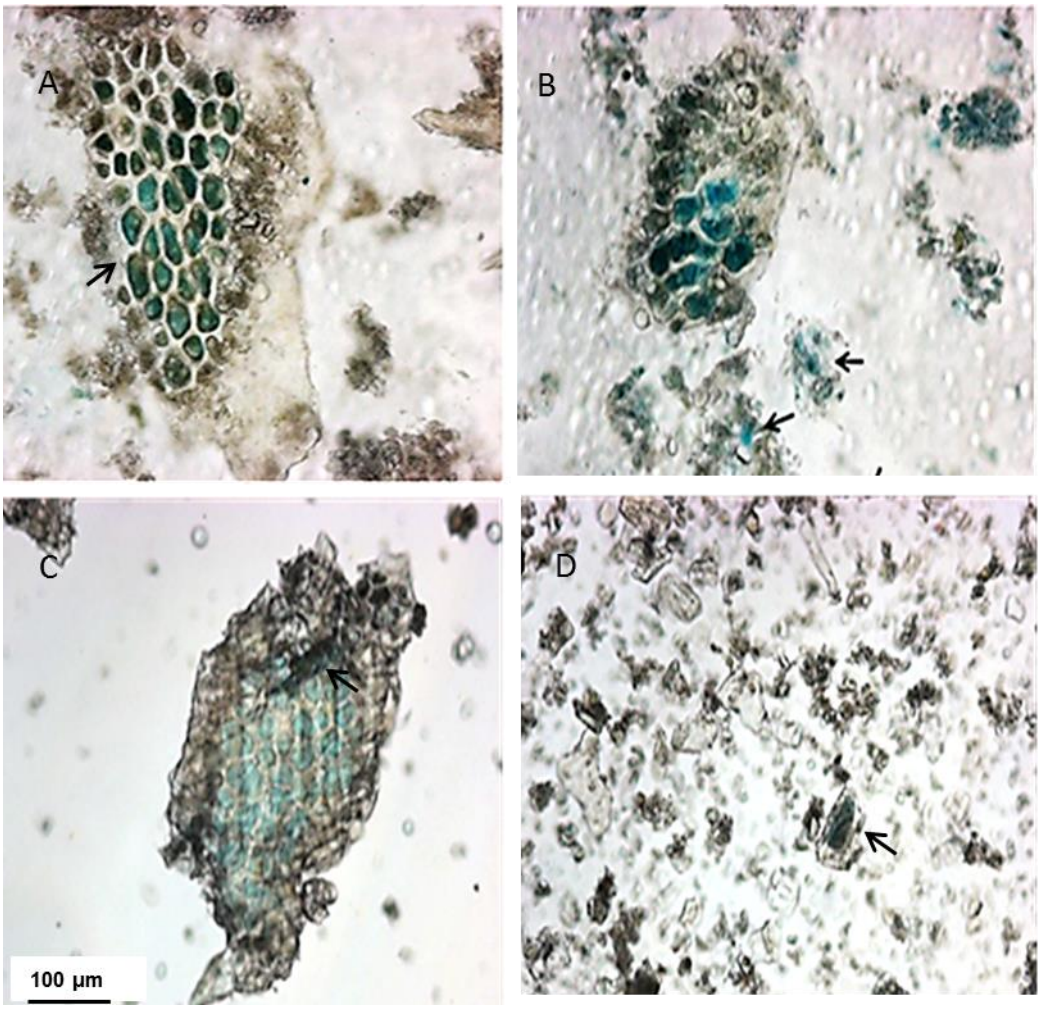
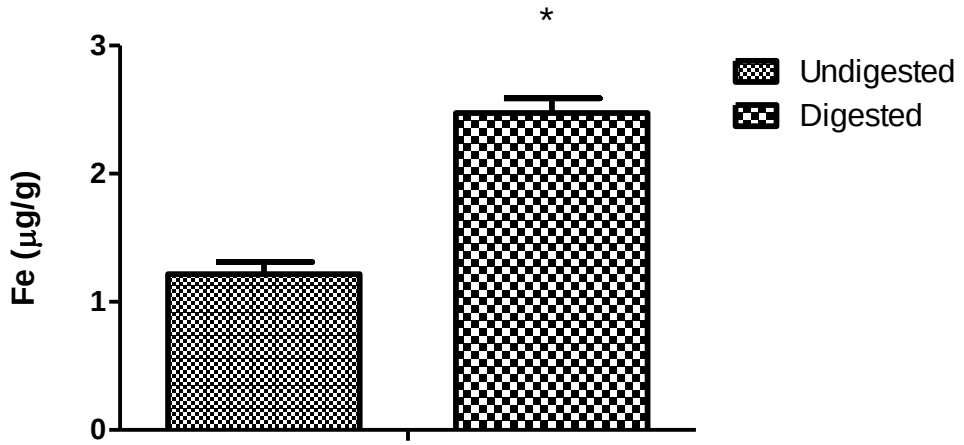


Fig 3. Location of iron in in wheat flour before (A) and after (B) digestion by driselase. Wholegrain wheat flour was digested by driselase (final concentration of 0.1%) at 37 °C for 6 h. Structure and localization of iron in whole aleurone flour (C) and micro-milled aleurone flour (D). Samples were treated with Perl's Prussian Blue solution and observed under microscope. Iron staining in intact aleurone in wholegrain and aleurone flour and in diffuse particle globules in digested wholegrain and micro-milled aleurone (arrows).

(A)



(B)

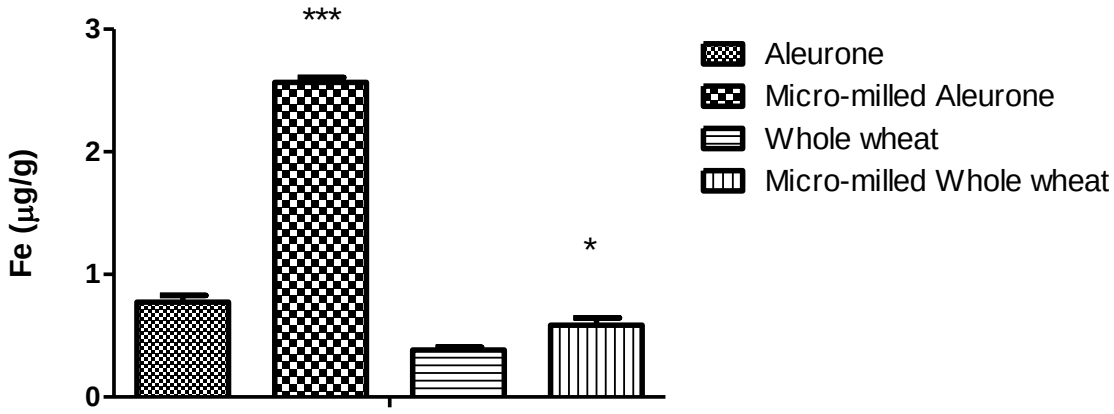


Fig 4. Iron solubility in wholegrain wheat flour after digestion with driselase (A). Iron solubility from standard- and micro-milled and aleurone and whole wheat flour (B). Data are means ± SEM, n=4-6, Comparison of means was analyzed by Student's t-test.* $P<0.0001$, (aleurone) and $P<0.05$ (wholewheat).

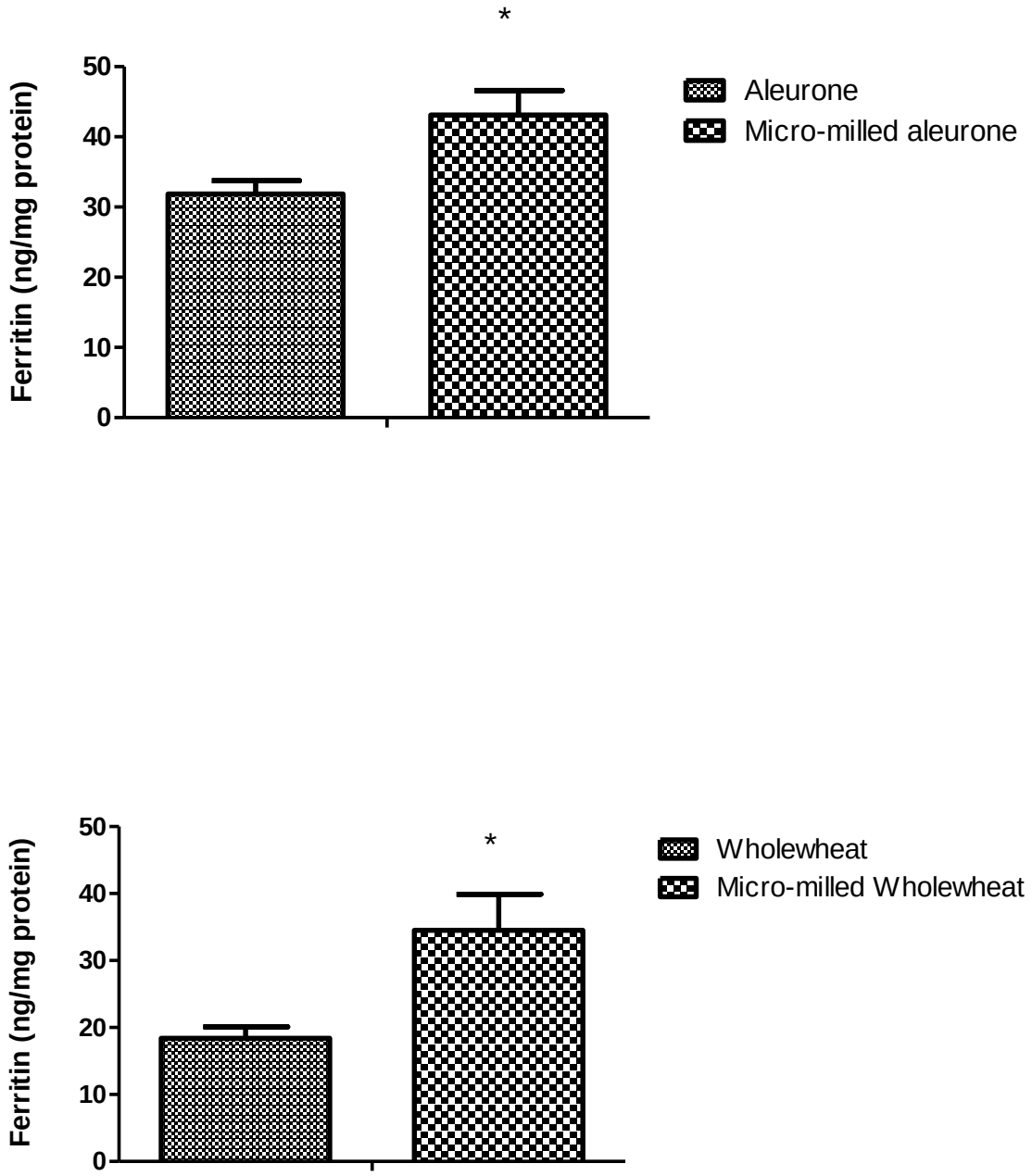


Fig 5. Iron bioaccessibility from standard- and micro-milled aleurone flour (a) and whole wheat flour (b) expressed in terms of Caco-2 cell ferritin synthesis. Data are means ± SEM, n=6, Comparison of means was analyzed using Student's t-test. * $P<0.01$.

Table 1: Mineral concentrations in wheat samples

Wheat fractions	Minerals (mg/ 100 g dry weight)					
	Fe	Mn	Zn	Ca	Mg	Cu
Aleurone	14.0±0.36	9.50±0.29	10.02±0.29	102±1.13	792±6.7	1.53±0.11
Aleurone(Micro milled)	12.7±0.05	6.81±0.11	8.15±0.18	91.0±0.09	660.1±0.41	1.30±0.04
Whole wheat	3.5±0.05	4.71±0.55	3.18±0.09	40.4±0.47	139.6±3.4	0.47±0.02
Whole wheat(Micro milled)	3.5±0.10	4.02±0.07	2.99±0.03	39.5±0.63	129.3±2.2	0.36±0.04

Values are means ± SE (n = 4)

TOC Graphic